PATENT
Attorney Docket No. 045636-5083

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Marche and al.

Confirmation No.: 7396

Art Unit: 1637

Serial No.: 10/581,814

Examiner: Samuel Woolwine

Filed: December 3, 2004

For: METHOD FOR QUANTITATIVE EVALUATION OF A REARRANGEMENT OR A TARGETED GENETIC RECOMBINATION OF AN INDIVIDUAL AND USES THEREOF

DECLARATION UNDER RULE 132

Commissioner of Patents
PO BOX 1450
Alexandria, VA 22313-1450

Dear Commissioner:

Now comes Nicolas Pasqual, who declares and states that:

1. I have the following degrees and qualifications: PhD in Biology and immunology, M.S. in Cellular & Molecular Biology, and a B.S. in physiology.

2. I have been employed by COMMISSARIAT A L'ENERGIE ATOMIQUE ET AUX ENERGIES ALTERNATIVES (CEA) for 3 years and I am now President, CEO and CSO of ImmunID Technologies (since 5 years).

3. I declare that I am experienced in the field of Immunology, immunogenetic, molecular biology and biology, as may be seen from the attached truncated curriculum vitae and the publication list attached hereto.

4. I am one of the named inventor of the above-identified Patent Application ("the '814 Application")

5. I have reviewed the Office Action dated October 30, 2009 and I declare the following :

6. Contrary to what was described in Pasqual and al. the instant method by combining the followings steps of:

- Amplifying a **long segment of gDNA** (having a size between a few hundred base pairs and several tens of kb in size) from a **human biological sample** by long multiplex PCR in which the elongation steps are performing at least for 10 minutes at 68°-72°C,
- Using at least one pair of primers in which (1) one of said primers V hybridizes upstream or at the 5'terminal end of a V gene of the variable domain of the α chain of TCR and (ii) the other primer J hybridizes downstream or at the 3' end of a gene J of the α chain of TCR,
- Using a DNA polymerase having a correction activity, and
- Separating the amplified DNA products by electrophoretic migration on a gel and detecting said amplified products directly on said gel by using a DNA-labeling agent during the migration and detecting after excitation in the UV range or another appropriate wave length or by using during amplification, labeled primers,

is able unexpectedly to improve the readability of the gels compared to the results observed in Pasqual and al. conditions, in which a Southern blot is compulsory in view to obtain readable results.

7. Tests were performed under my supervision and confirm that it is only by combining the specific features of the instant invention that it is possible to avoid the use of the Southern blot step which is not adapted to routine tests.

8. Exhibit A : Pasqual et al. conditions
In the conditions of Pasqual et al., it was possible, at the time of the publication of Pasqual et al., to observe and resolve 4 to 5 rearrangements by multiplex PCR, only when the DNA was transferred on a membrane and radioactive probes were used (figure 2A), whereas in figure 1A, which corresponds to results obtained directly on a gel, only smears (which cannot be interpreted) are observed.

In these two figures (1A and 2A) 6 TCRAD V genes have been studied (V2, V12, V19, V20 and V101).

The results observed in figure 1A are not due to a coloration problem since the size markers (right lane of the gel in figure 1A) are clearly visible.

It emerges from said figures that in the conditions of Pasqual et al, it is not possible to measure the combinatorial diversity of the immune repertoire TRAV-J, directly on a gel using a fluorescent detection. In such a case, the very weak "specific signal" is hidden in a smear of non specific signal also very weak (see figure 1A). Therefore it is not possible to detect a V-J rearrangement directly after PCR. The only possibility, in the conditions of Pasqual et al. , to obtain a detectable signal, was to perform a Southern blot using radioactive probes (see figure 2A).

On figure 2A, the black blots correspond to the rearrangements detected by the radioactive probes used in the Southern Blot.

Such a method is very long to carry out, costly and risky:

. Transfer of the amplicons to a membrane (>4h)
. Fixation of the DNA on the membrane (some minutes)
. Labeling of the probes (20-60 minutes)

. Incubation in a hybridization roller (on the membrane (> 2h)
. Washings (30-120 minutes)
. Exposure of the membrane in a cassette in view to retrieve the radioactive signal (> 4H-several days)
. Reading the cassette (15 minutes).

It is not adapted to routine diagnostic tests.

9. Exhibit B : Pasqual et al. conditions

Method of Multiplex PCR assay:

Briefly, using an upstream primer specific for a given Vx family and a downstream primer specific for a given Jy segment, the multiplex PCR assay allows the detection of a Vx to Jy rearrangement as well as that of Vx genes to a limited set of 5' J segments spanning from Jy to Jy-4 position.

Amplifications were performed with 1.3 unit/reaction of Expend High Fidelity PCR system (Roche Diagnostics, Meylan, France)

The cycling conditions were 5 min at 94°C, 26 cycles of 1 min at 94°C, 1 min at 58°C, 6 min at 72°C, and one cycle of 10 min at 72°C. In these assay conditions, maximum amplicon size was approximately 5kb.

Quality control PCR using a non rearranged gene P53.

Normalization of the quantity of DNA in each reaction was determined by amplification of p53 gene **as a non-rearranging gene** in the same PCR run.

PCR is done using P53 specific primers. Primer P53 sens X6.5 : ACAGCGTGGTGGTACCTTAT, and Primer P53 anti-sens X7 : CACATGTACTTGTAGTGGATGG.

Migration : 10 µL of amplicon + 2 µL of loading dye is loaded on agarose Gel (1,5%) + ethidium bromide. Migration at 150V during 3hours. UV excitation in a Vilber Lourmat + camera box.

Results on Gel

P53 normalization: figure 3B

Direct detection of P53 gene (after UV excitation) in each sample indicates a good normalization of DNA quantity for each sample F18, F19, F20, J0, J1 and J28.
This information confirms that gDNA quantity and quality is good, thus rearrangement detection can be performed on these samples.

Secondarily this result indicates that Gel coloration is good and if no rearrangement can be directly detected on gel it is not a matter of gel coloration and UV excitation.

Rearrangements: figures 1B and 2B

Smear detection (after UV excitation) and quasi non distinguished V-J rearrangements **on Gel** for both ADV19 (figure 2B) and ADV20 genes (figure 3B) on J56, J48, J40, J33, J27, J16, J9 and J2.
It is compulsory to perform an additional Southern Blot with internal radioactive probe (figures 4B and 5B).

Results are interpretable only on Southern blotted membrane (figures 4B and 5B)

Detection of ADV19 rearrangements on central and distal J genes at J1 and J28: figure 4B
Detection of ADV20 rearrangement on proximal J genes at F19, F20, J0, J1, and J28 figure 5B.

Conclusions

The results observed are not due to coloration problem since the size markers (right lane of the gel in figures 1B and 2B) are clearly visible.

Furthermore whereas in figures 1B and 2B smears are obtained, in figure 3B, good results are obtained directly on a gel, when in the same conditions, as in figures 1B and 2B, gene P53 is used.

Thus in the conditions of document Pasqual and al., a step of Southern blot is compulsory in view to characterize the rearrangements.

On the contrary, in the conditions of the instant invention, it is possible to obtain a map of the immune repertoire with strong signals, this allowing a direct detection on gels with the possibility of using such a method in routine diagnostic tests, in less than one day.

10. Exhibit C: Instant invention conditions

Figure 1C illustrates the results obtained from a human sample, with the method according to the instant invention.

More precisely:

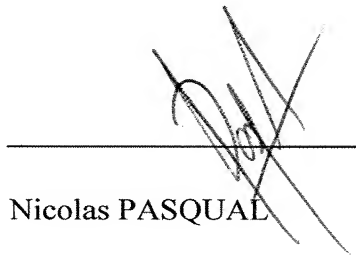
Multiplex PCR assay: multiplex PCR is performed with V1 (=ADV19) and V8 (ADV18) upstream primers on J5 and J33 gene downstream primers as described in the present invention.

Migration : 10 µL of amplicon + 2 µL of loading dye is loaded on agarose Gel (1,5%) + ethidium bromide. Migration at 150V during 3hours. UV excitation in a Vilber Lourmat + camera box.

Results : Direct UV detection of V1 and V8 rearrangements on J5 and upstream J genes as well as J33 counterparts.

Coloration with SyberGreen (or BET) is possible and effectively allows the detection of the rearrangements directly on the gel.

11. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief to be true; and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the Application or an Patent issuing there from.



Date: 19/4/2010

Nicolas PASQUAL

Exhibit A

Figure 1

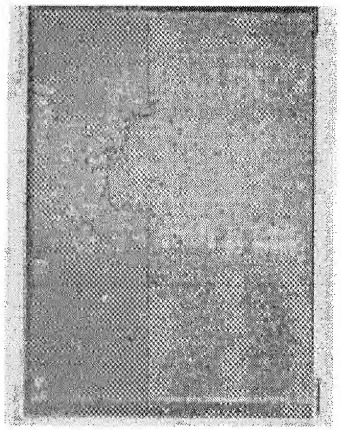


Figure 2

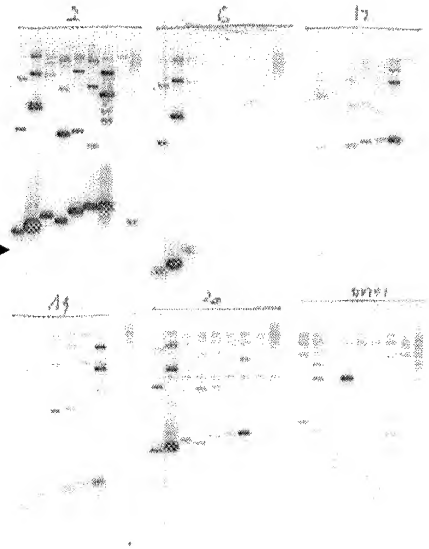


Exhibit B

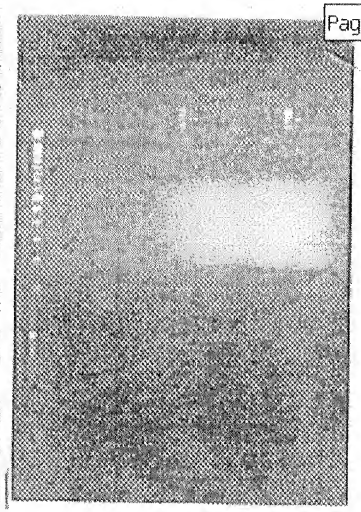


Figure 1

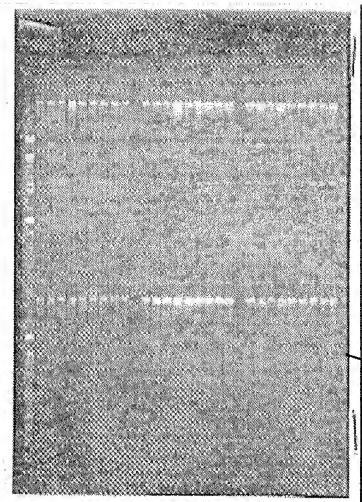


Figure 2

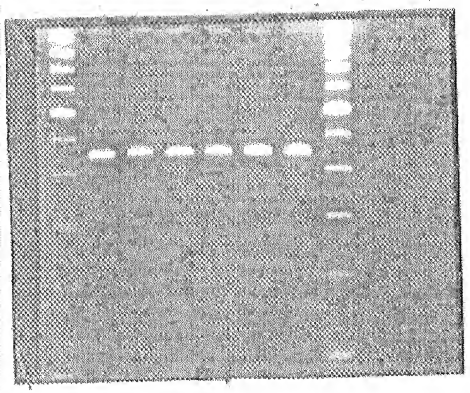


Figure 3

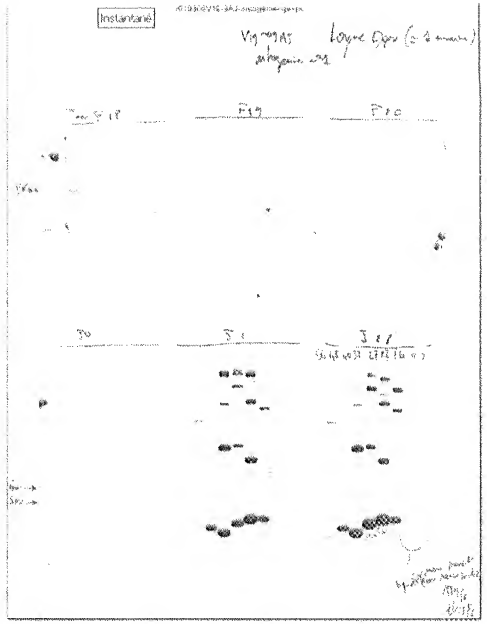


Figure 4

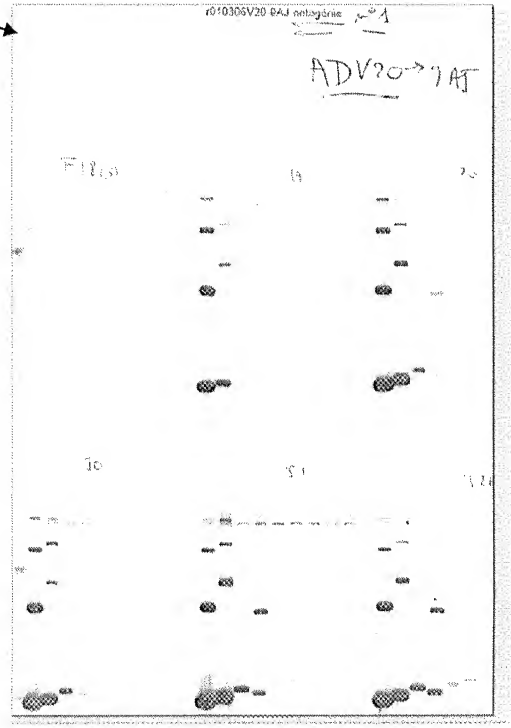


Figure 5

Exhibit C

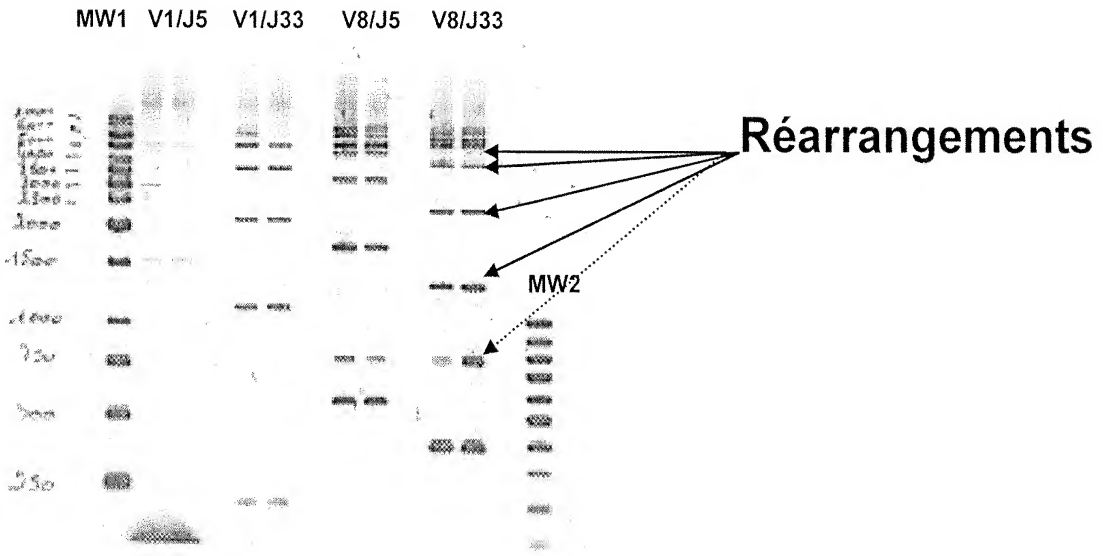


Figure 1